ABSTRACT

ROPER, WAYNE ROBERT. *Rhizobium leguminosarum* Strain Combination Effects on Nodulation and Biological Nitrogen Fixation of *Vicia villosa*. (Under the direction of Dr. Owen Duckworth and Dr. Julie Grossman).

Hairy vetch (*Vicia villosa*) is an important legume cover crop used to improve soil fertility in cropping systems. The soil bacterium *Rhizobium leguminosarum* specifically nodulates vetch roots and fixes atmospheric nitrogen (N\textsubscript{2}) into ammonia (NH\textsubscript{3}) that is assimilated into plant tissue. Many crop producers inoculate fields with rhizobia to enhance biological nitrogen fixation (BNF), but utilization of more effective inoculant strains is limited by competition from resident rhizobia. Improved BNF in cover crop legumes could enhance N fertility and reduce fertilizer requirements in organic systems. We evaluated the ability of four rhizobia strains to nodulate and improve vetch growth as individual and combined inoculants. Plants were inoculated with equal ratios of one to four strains and grown under controlled conditions for 46 days. After harvest, shoot biomass, N content, nodule number, nodule mass, and occupancy were measured. Negative-N and positive-N (NH\textsubscript{4}NO\textsubscript{3}) controls averaged 6.3 and 83.2 mg total shoot N, respectively. Average total shoot N of inoculated vetch treatments was between that of the uninoculated controls. Nodule number, total nodule mass, and BNF efficiency (N mass per nodule mass) were not significantly different between individual strains, but on average, two strains produced more BNF than others. Several treatments resulted in more BNF from multi-strain inoculants rather than single-strain inoculants, suggesting that BNF in vetch was not limited to the efficiency of individual strains. We also observed that nodule occupancy as evaluated by PCR DNA fingerprinting with BOX-A1R was not correlated with BNF capacity. For example, the strain with highest competitiveness for nodule occupancy (NCSU435) had the lowest BNF capacity. In general, BNF produced by rhizobia in multi-strain inoculants was greater than single-strain inoculants, but changes in BNF were not attributable to relative nodulation by a single strain. Overall, this suggests that improving rhizobia diversity may be a critical factor for improving BNF.
Rhizobium leguminosarum Strain Combination Effects on Nodulation and Biological Nitrogen Fixation of Vicia villosa

by
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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science

Soil Science

Raleigh, North Carolina

2015

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DEDICATION

To the advisors and cohorts who had confidence in me throughout the project and provided support where it was needed. Also to my family who remained proud of me throughout this experience.
Wayne was born in Detroit, MI, USA where he grew up surrounded by one of the most industrialized and urban areas in the country. In Detroit there were several environmental issues stemming from industrial activity and property neglect and this inspired him to seek an education in the environmental sciences. During his undergraduate studies he had many opportunities to learn about ways to balance land use with sustainable management. After working with landscape architects on construction and design of sustainable public spaces he took a more scientific approach and begin analyzing the soil itself. His increased knowledge of chemistry and botany resulted in a desire to garden and seek more opportunities in plant and soil sciences. A couple summers of organic gardening and surveying the natural area of the Henry Ford estate in Dearborn, MI was fun, but in order to achieve more as a researcher he began graduate school at the Soil Science department of North Carolina State University where he studied biological nitrogen fixation of legumes and rhizobia.
ACKNOWLEDGMENTS

Dr. Julie Grossman For seeing the potential in me, inviting me on as a graduate student, and providing mentorship in the ways of proper scientific research

Dr. Owen Duckworth For accepting me as a student and providing excellent tutelage in data analysis and academic writing

Dr. Dan Israel and Dr. Michael Hyman Members of my advisory committee who steered me towards the proper information on legumes and microbiology

Sarah Seehaver The technician of the Grossman lab whose excellent teaching, organization, and planning helped me complete most of the research

Dr. Thanwalee Sooksa-nguan, Dr. Mary Parr, Nape Mothapo, Amanda Roth, Peyton Ginakes, and Sean Blozies Members of the Grossman lab who provided their knowledge and assistance to help me complete the research

USDA-NIFA Funding for the research through the organic transitions program

Lisa Lentz Analysis of plant tissue samples through the Environmental and Agricultural Testing Service

Dr. Consuelo Arellano Statistics consulting

Soil Science faculty, staff, and students For being supportive and friendly whenever a fellow graduate student needed someone to talk to or needed help

College and Agriculture and Life Sciences Awarded me additional funding through the Dean’s Graduate Research Assistantship

My parents and family They were always concerned and kept encouraging me to apply myself in order to get more out of life.
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Chapter 1: Legumes as Cover Crops and the use of Inoculation to Improve Biological Nitrogen Fixation - A review

1.1 Context of Work

In the year 2014, world population reached over 7 billion people, and is predicted to exceed 10 billion people by 2050. This growth will place a greater strain on food supply (United Nations Department of Economic and Social Affairs, 2013) and maintaining soil fertility is of great importance as food producers try to address increasing food demand. Historically, technological advances and improved understanding of natural processes has helped agriculture keep pace with growing population and increasing food demand, but innovation has slowed while population growth continues. Conventional means of agricultural production are unlikely to satisfy future food demand unless land becomes more fertile (Food and Agriculture Organization, 2011), and therefore it is becoming more important to reduce the strain on natural resources such as water, soil, and biodiversity by adapting improved methods of sustaining soil fertility.

Cover crops are herbaceous plants used to manage soil and have been shown to result in beneficial outcomes such as soil stabilization and reduced leaching of essential nutrients (Clark et al., 1995). Cover crop roots can also retain nutrients that would otherwise leach to deeper depths (Wyland et al., 1996). Additionally, they suppress moisture loss and weed growth by providing groundcover (Lichtenberg et al., 1994; Blackshaw et al., 2001). Because cover crops are not harvested, plant residues are tilled into soil or allowed to decompose in place, which results in increased soil organic matter, soil moisture, and plant available nutrients (Wander et al., 1994; Hermawan and Bomke, 1997; Larsen et al., 2014). Despite the benefits of cover crops, only about 3% of arable land surveyed in the USA regularly includes them in plantings (USDA, 2012), but new
initiatives are being created to highlight their importance (National Working Group on Cover Crops and Soil Health, 2014).

Plants require many different nutrients for optimal growth, but nitrogen (N) is often the nutrient that limits plant growth in soils (Attiwill and Adams, 1993). Although the majority of N added to crops now comes from synthetically produced N (Smil, 2011), a significant portion is derived from biological nitrogen fixation (BNF), the process by which some bacteria grow by assimilating N from atmospheric N\textsubscript{2} (Kouchi, 2011). Because manufacturing synthetic N fertilizers is energy intensive and expensive, utilizing cover crops that increase available N through BNF provides another benefit to soil fertility and crop production.

### 1.2 Legume cover crops and biological nitrogen fixation

Perhaps the most commonly grown leguminous crop is soybean, yet other plants, such as alfalfa, sweet pea, and peanuts, are also popular commercial legume crops that can fix N. Legume species, especially soybean, are usually included in crop rotations to restore soil N that is lost to harvested crops and other processes (Nemecek et al., 2008). Although not serving as cover crops specifically, commercial legumes are beneficial because a substantial portion of the N stored in their residue is assimilated from BNF, which reduces the need for other N fertilizers. Cover crops are not harvested, so leguminous cover crops are able to contribute more N than commercial species, and therefore have greater potential to improve soil fertility.

Symbiotic fixation of N occurs within legumes because they form a symbiotic relationship with soil dwelling bacteria that are capable of BNF called rhizobia (Giller, 2003; Prell and Poole, 2006). Rhizobia are common in most soils and can forage in environments without the aid of their symbiotic partner legume (Ahmad et al., 1984). There are ecologically diverse rhizobia species.
associated with a wide variety of habitats and host plants (Lafay and Burdon, 2006; Han et al., 2009; Sene et al., 2012), but their relationships with host legumes are generally similar. Symbiosis begins when legumes exude flavonoids into soil, indicating their presence to nearby rhizobia. Rhizobia in the root zone of the legume exude their own signaling compounds in the form of nod factors, which are polymers comprised of fat, sugar, and protein (Oldroyd, 2013). The nod factors stimulate legume root hairs to allow rhizobia to penetrate into root tissue and, once inside plant cells, rhizobia multiply and produce nodules on the roots of the host plant. Upon successful nodulation of legume roots, rhizobia inhabit nodules with a low oxygen environment from which they utilize atmospheric dinitrogen gas (N₂) to produce ammonia (NH₃), which is used by the host legume. In exchange for reduced N, the host plant provides nodulating rhizobia with sugar to use as a carbon source. Symbiosis is beneficial for individual rhizobia because rhizobia occupying nodules may be rewarded with more sugar depending on the amount of N supplied to the host legume.

Several factors impact the rate of BNF, including legume species, rhizobia species, rhizobia population density, and soil nutrient availability. Multiple environmental factors affect choice cover crop species (i.e., white clover (Trifolium repens) (Campillo et al., 2005), Austrian winter pea (Pisum sativum subsp. arvense) (Parr et al., 2011), and crimson clover (Trifolium incarnatum)(Evers and Parsons, 2011)), and usage is most often determined by climate and desired crop rotation. Legume cover crop N is often produced in quantities that can satisfy a substantial portion of the N required for optimum yields (Tian et al., 2000; Cicek et al., 2014) and, to encourage wider usage, various legume crops are credited with contributing residual N for successive crops (Rodale Research Center, 1988; Beckie and Brandt, 1997; Jensen et al., 2010). Although legume cover crops alone may not supply all N requirements for desired productivity (Parr et al., 2011), they can still supplement
and reduce the need for other fertilizers that may overload soil with excess nutrients (Ouyang et al., 2013).

1.3 Hairy vetch

When planting a cover crop, it is preferred that the species has low seed cost and broad vegetative coverage to optimize its utility while minimizing expenses. One legume species with versatile use is hairy vetch (*Vicia villosa*), which is an annual that overwinters in multiple hardiness zones (4 to 6) ranging from Wisconsin to South Carolina in the USA. Vetch can also grow in a variety of conditions, including dry soil and pH ranges 5.0–7.5, allowing it to survive in a wider range of environments. Vetch planted in fall will lie dormant during winter and continue growing in spring as a prostrate groundcover that suppresses weeds and reduces moisture loss as the planting season for other crops approaches (Duke, 1981). Vetch stems are viny and often several feet long at maturity when groups of stems spread along the ground to form masses of vegetation. Coverage provided by vetch is extensive and cost effective enough to rival other types of soil coverage (Kelly et al., 1995). Vetch flowers in late spring which allows ample time for it to last as a cover crop to be tilled into soil or killed in time to decompose before the next planting. Residue from vetch plants typically has between 4–5% N concentration and is among the highest N concentrations for common legume species used as cover crops (Degregorio et al., 1995; Lawson et al., 2012; Mothapo et al., 2013). By comparison, clover often has 2–3% and pea has 3–4% N concentration (Hargrove, 1986; Parr et al., 2011).

Rhizobia are generally present in most soils (Moawad and Bohlool, 1992; Amarger, 2001), but there is host specificity in rhizobium-legume symbiosis that may result in insufficient nodulation in the absence of compatible symbionts (Provorov, 1998). Hairy vetch is nodulated by the rhizobium
species *Rhizobium leguminosarum* biovar *viciae* (Rlv) (Laguerre et al., 2003). Rlv nodulates plants in the viciae tribe that, along with common vetch (*Vicia sativa*), includes faba bean (*Vicia faba*) and peas (*Pisum sativum*). It is not clearly understood why rhizobia are selective for certain legume species, but co-evolution and diversity of legumes and rhizobia is well researched (Dresler-Nurmi et al., 2009; Rogel et al., 2011). Vetch nodulation induced by Rlv results in indeterminate nodules that grow longitudinally outward as bacteria inside them multiply (Masson-Boivin et al., 2009). Typically, nodules produced by rhizobia grow larger as the host plant grows, but with a poorly performing rhizobia population, nodules on vetch can fail and cease N fixation (Schumpp and Deakin, 2010). Absence of nodule growth on vetch roots is often a sign of undesirable soil or a scarce rhizobia population. It has been shown that when hairy vetch is planted in a soil without a history of vetch cultivation, BNF, nodule mass and nodule number are reduced when compared to soils with recent vetch cultivation (Mothapo et al., 2013). Without continued maintenance of a compatible rhizobia population, producers using legume crops may not maximize N yields from BNF even if using a productive legume like hairy vetch.

### 1.4 Rhizobia inoculation and its impacts on BNF

To ensure optimum BNF, rhizobia populations are often enhanced by inoculation, the addition of specific rhizobia to an environment to increase their population (Deaker et al., 2004). After inoculation, the rhizosphere is supplemented with a population of compatible elite rhizobia strains observed as optimum N fixers with a particular host legume species. Rhizobia inoculation is often accomplished by culturing rhizobia in media that can be affixed to legume seed coats, mixed with peat, or added directly to a solution (Herridge et al., 2014) and then spread onto the soil where the inoculant rhizobia population is subsequently increased. Seed inoculation tends to work best
after years of cultivation of the desired legume, but may not be sufficient for establishing new populations of compatible rhizobia, which is what peat and powder inoculants are more commonly used for when they are applied with legume seeds (Herridge et al., 2014). Mixtures of rhizobia species are added to inoculants, and after the inoculants are applied to soil, the rhizobia population increases when the preserved rhizobia revive and multiply. Vetch, pea, and lentil inoculants are usually mixed with *Rhizobium sp.*, whereas soybean inoculants include *Bradyrhizobium sp.*, soybean’s symbiotic partner (Abaidoo et al., 2007). The current variety of commercial inoculants is small, but could grow as legume cover crops are more frequently utilized.

Studies dating back decades noted low survival rates of rhizobia on legume seed and expressed concern over the viability of seed inoculation (Brockwell et al., 1975). Development of more efficient inoculation products has led to new rhizobia isolates and application techniques. Modern seed inoculants included rhizobia cells preserved in degradable polymer coatings and different seed storage recommendations (Bashan et al., 2014). Improved survivability of rhizobia in inoculants is especially important for newly cultivated land, where rhizobia inoculation can be helpful for establishing new populations. Legume seeds can be sold coated with rhizobia, but often inoculants are added to seed just before planting to ensure the inoculant rhizobia population is fresh and active.

The degree to which BNF is improved by inoculation has been measured in several ways. A common approach is the comparison of uninoculated plant N content to that of inoculated or fertilized plants of the same species to determine the impact of BNF on growth and N assimilation (Hardarson and Danso, 1993). Alternately, a non-nodulating variety of the legume species can be used because it can be grown in the same soil as nodulating legumes and provide direct
comparisons (Oikawa et al., 2013). However, non-nodulating varieties of legumes are uncommon in most cover crop species and therefore not frequently used. Quantitative measurements of BNF can be accomplished with stable elemental isotopes (Unkovich and Pate, 2000), relying on the existence of atmospheric N\textsubscript{2} as \textsuperscript{14}N and \textsuperscript{15}N isotopes in a natural ratio. The isotopes have a natural abundance in soil that can be used as a reference, but sometimes \textsuperscript{15}N labeled fertilizer is added to soil. The assimilation of \textsuperscript{15}N in a reference plant can be measured, and because BNF has a preference for \textsuperscript{14}N isotopes, \textsuperscript{15}N is diluted in plants performing BNF. The amount of \textsuperscript{15}N detected in plants compared to \textsuperscript{15}N labeled soil provides a relative amount of BNF as total N assimilation. Calculating BNF using uninoculated plants and N isotopes typically provides the same results (Martensson and Ljunggren, 1984; Urquiaga and Boddey, 1987), and the two methods may be used interchangeably.

1.5 Root nodulation and competition within rhizobia populations

Soil inoculation is expected to positively impact legume production by introducing an efficient N fixing strain to the germinating legume seed to outcompete resident soil rhizobia for nodulation sites (Carter et al., 1994; Kurchak and Provorov, 1995). Soils with small populations of compatible rhizobia often see greater plant response to inoculation than soils with large populations of compatible rhizobia, and increases in N yields from BNF will likely be larger (Thies et al., 1991). Tests evaluating legume cover crop inoculation effectiveness have been successful in both field and laboratory conditions resulting in increased BNF (Laguerre et al., 2003; Spriggs and Dakora, 2007), but long term benefits of continuous inoculation are unclear. Once a desired population of rhizobia is established and N yields from BNF are stable, the value and effect of inoculation may decrease (Furseth et al., 2012). Possible reasons for this reduction in impact are that larger and more diverse
resident rhizobia populations may compete with inoculant rhizobia for plant nodulation or inoculant rhizobia may not grow well in new environments.

Resident rhizobia populations often outnumber introduced strains and can advantageously colonize the rhizosphere. Relative abundance among many other compatible rhizobia is only one of many determining nodulation factors (Vlassak and Vanderleyden, 1997), but it may be the most relevant factor limiting nodule occupancy by inoculant strains besides host compatibility. It is unknown if competition for nodule occupancy exists between rhizobia or if nodulation occurs by chance (Friesen, 2012). Evolutionarily, it is expected that efficient rhizobia genotypes would most often be found in nodules due to favorable selection by the host plant (Friesen, 2012). This type of selection would be beneficial to application of rhizobium inoculants containing highly efficient strains, but the mechanism by which host legumes may reject strains in favor of symbiotically efficient rhizobia has not been identified (Kiers et al., 2003). Observations of nodules failing long before host maturity suggest that some inefficient nodule occupants are rejected (Simms et al., 2006). Conversely, other research has found no relationship between relative nodulation and BNF supplied by rhizobia found in nodules (Gubry-Rangin et al., 2010). Because relationships in the rhizosphere are complicated by several variables, it may be possible for effective rhizobia to provide host legumes with sufficient N while less effective nodulating rhizobia also receive the benefit of symbiosis without contributing as much energy to BNF (Friesen, 2012). The potential for some rhizobia to exploit symbiosis creates ambiguous relationships between N fixation and nodule occupancy by strains. Continued improvements of inoculant technology require better understanding of the rhizobium-legume symbiosis and increased ability to assess the success rate of applied inoculant strains competing for nodulation. One approach may be identifying rhizobia
nodule occupancy within a range of strain types, and quantifying how BNF is affected by changes in nodule occupancy.

1.6 Rhizobia Identification

Different techniques for identifying rhizobia strains recovered from nodules have been utilized over time (Thies et al., 2001). Culturing and serotyping methods used to classify rhizobia were standard until the 1990s, but as technology improved, molecular genetics approaches that analyze rhizobia DNA became the dominant techniques used to identify and track rhizobia strains found in nodules (Thies et al., 2001). Bacterial DNA subjected to polymerase chain reaction (PCR) amplification can produce strain-specific markers with the use of PCR primers targeting specific regions of rhizobial and bacterial genomes. Many PCR primers exist to accomplish this, but the standard approach used to characterize rhizobia is the repetitive extragenic palindromic technique, or rep-PCR (Schneider and Bruijn, 1996).

With rep-PCR, DNA in the 16S–23S ribosomal gene sequences containing palindromic repeats of nucleotides is amplified to produce several different sized amplicons comprising the entire DNA fingerprint of a bacterium. Rep-PCR is useful for identifying rhizobia strains because the amplified region of DNA is polymorphic, meaning that fingerprints have a degree of uniqueness that can be traced back to the original strains after inoculation and extraction of DNA from nodule tissue. The most common rep-PCR techniques for rhizobia involve the BOX and ERIC PCR primers and their iterations (Nick et al., 1999). Given any collection of bacterial DNA, rep-PCR primers can generate signature DNA fingerprints that collectively reveal the relative similarity or diversity of the population. Other PCR DNA regions, including those associated with genes for producing nod factors
Diversity in rhizobia populations has become a popular research topic due to the ability of DNA fingerprinting to quickly identify genetic variability amongst many rhizobia strains of the same species. Links between rhizobia nod genotypes have been found, as well as links between legume and rhizobia populations, providing evidence for legume host preference for rhizobia genomes. However, no convincing links have been made between rhizobia species genotypes and the ability to nodulate (Amarger, 1981). Some studies have found few inoculant genotypes present in nodules after field application (Malek et al., 1998; Denton et al., 2002; Seehaver, 2013) whereas others successfully identified a significant number of nodules containing inoculant rhizobia DNA (Denton et al., 2003; Beyhaut et al., 2006). Results from field studies tend to differ from more controlled laboratory studies where inoculant strains have more success competing for nodulation against resident rhizobia (Malek et al., 1998; Laguerre et al., 2007). Such research suggests that resident rhizobia may decrease BNF from efficient inoculant rhizobia strains because resident rhizobia occupy more nodules.

To reduce the number of variables complicating evaluations of rhizobia competition using DNA analysis, the number of strains used to assess nodule occupancy under controlled conditions is often minimized (Malek et al., 1998). Using only two strains may reveal an occupancy pattern between those two strains, but it fails to address concerns of opportunistic nodulation in the presence of multiple rhizobia that may be better nodulators or more efficient N fixers. To account for differences in nodulation in the presence of many other strains, inoculants should use combinations of multiple strains that can be distinguished by DNA fingerprinting. Rhizobia strains
that consistently occupy more nodules and improve BNF could likely do well against resident rhizobia and become established as major nodulating strains in soil (Hebb et al., 1998). Genetic identification techniques can be used to determine relative nodule occupancy of inoculant strains applied to vetch in competition with other rhizobia strains.

1.7 Summary

It is argued that agricultural productivity must increase in order to satisfy growing food demand, but intensifying land use is likely to cause detrimental effects on water, ecosystem, and soil health. Cover crops can reduce the impacts of production on soil by minimizing erosion, suppressing weed growth, and retaining nutrients, but with N being the most limiting nutrient in soils, developing a sustainable method of managing its availability is crucial. The rhizobium-legume symbiosis provides N for maintaining soil N fertility, and hairy vetch is among the most commonly used cover crops.

Because legumes form effective symbiosis with certain rhizobia species, it may be necessary to inoculate soil with Rlv, vetch’s symbiotic partner, to optimize BNF. Studies have shown that BNF can be deficient in soils without a recent history of the desired legume (Mothapo et al., 2013), and that inoculation can be an effective method of maintaining rhizobia populations (Trabelsi et al., 2011). However, new research has become increasingly focused on selection of high performance rhizobia that effectively compete with resident rhizobia for nodule occupancy of host legumes. Standard practice evaluates rhizobia performance after single-stain application to the host, but in practical use, the effect of an inoculant can change when faced with competition from other microbes. Techniques like PCR DNA fingerprinting have enhanced characterization of rhizobia genetic diversity as well as the ability to generate strain specific DNA markers used to track rhizobia
strains and match them to rhizobia DNA extracted from legume nodules. These techniques provide more accurate assessments applied inoculant rhizobia strains and their ability to compete with resident rhizobia for nodule occupancy of legume hosts.

Considering the many factors affecting the rhizobium-legume symbiosis, our research objectives are to (1) determine if Rlv strain combinations have additive effects on BNF and nodulation of vetch based on individual strain characteristics, and (2) assess whether relative nodule occupancy of Rlv strains regulates BNF capacity of vetch. We hypothesized that adding less efficient rhizobia to inoculants would decrease BNF compared to inoculants with more efficient rhizobia and that changes in BNF for one inoculant compared to others would accurately reflect changes in nodule occupancy by individual rhizobia strains. Hairy vetch and Rlv may make a productive legume cover crop and inoculant combination, but the viability of inoculation with specific Rlv strains must first be evaluated. As cover crops gain broader application, this information would be useful to producers looking to optimize BNF from their vetch cover crop.

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Chapter 2: Strain combinations affecting BNF of hairy vetch

2.1 Introduction

Cover crops are used for sustainable management of soil health and fertility. They benefit agroecosystems by reducing erosion through soil stabilization (Clark et al., 1995), providing weed suppressing ground cover (Blackshaw et al., 2001), limiting nutrient leaching (Wyland et al., 1996), and adding plant residues that can mineralize into nutrients for successive crops (Ranells and Wagger, 1997). In addition, leguminous cover crops can enhance fertility by providing nitrogen (N) to plants through biological nitrogen fixation (BNF), the conversion of atmospheric N (N\textsubscript{2}) into plant available ammonia (NH\textsubscript{3}) inside of legume root nodules created by symbiotic bacteria called rhizobia (Prell and Poole, 2006). This makes legumes desirable ‘green manures’, plant residues meant to provide mineral nutrients. Legume cover crops have especially been helpful for those who practice organic and sustainable methods of production (Clark, 2007). Despite their many benefits, only about 3% of farmland in the US is reported as using cover crops in rotations (USDA, 2012). Several agencies have intensified advocacy for increased legume cover crop usage and, as a result, many legume species have been investigated for their potential to enhance N fertility (Herridge et al., 2008).

Hairy vetch (Vicia villosa) is a widely adapted legume cover crop in the United States. Vetch is a winter hardy cover (hardiness zones 4 to 6), and is typically planted in late summer or early fall to provide N for the next growing season. Additional benefits of vetch include a greater average N concentration compared to several other cover crop legumes (Degregorio et al., 1995; Lawson et al., 2012; Mothapo et al., 2013), fast mineralization of residues (Azam et al., 1993; Brandsæter et al., 2008), and extensive groundcover (Teasdale et al., 2004; Campiglia et al., 2010). Studies have shown
vetch to have greater N yields (100 to 135 kg N/ha) compared to Austrian winter pea (*Pisum sativum subsp. arvense*) and white clover (*Trifolium repens*), two other common legume covers (Lichtenberg et al., 1994; Parr et al., 2011). Additionally, when vetch is planted after a late summer or early fall harvest, it can over-winter and reach its maximum N supply by the next spring season.

To obtain optimum benefit as a cover crop, it is desirable to maximize BNF from vetch and its symbionts. In vetch, BNF is initiated when *Rhizobium leguminosarum* biovar *viciae* (Rlv) bacteria penetrate root tissue and N fixing nodules develop on host roots. If Rlv is sparsely populated in soil, a newly established vetch cover crop is unlikely to form adequate nodule mass to produce optimal N from BNF (Mothapo et al., 2013). A common practice in legume cultivation is to inoculate legume seeds or soil (via liquid or peat) with compatible rhizobia strains to enhance nodulation and BNF from the crop. Researchers have reported improvements in yield after inoculation (Buttery et al., 1992; Carter et al., 1994; Kurchak and Provorov, 1995; Denton et al., 2013), and even after establishing a large population of compatible rhizobia, inoculation can be used to maintain a diverse rhizobia population (Trabelsi et al., 2011).

One problem with inoculating cover crops has been determining the effectiveness of rhizobia strains for BNF. Inoculant rhizobia compete with resident strains for resources and root nodulation. While the effect that a single-strain inoculant has on legume BNF under controlled conditions is measurable (Ballard et al., 2004), when a single rhizobium strain nodulates roots in combination with several other rhizobia, it is difficult to attribute improvements in nodulation and BNF solely to the presence of a single applied strain because tracking the rhizobia most successful at symbiosis has historically been difficult (Hirsch, 2005). The application of modern molecular biology techniques to rhizobia studies has made their identification easier (Thies et al., 2001; Sarita et al.,
Coupling PCR amplification of rhizobia DNA from nodules with identification by DNA fingerprinting or genetic sequencing allows for advanced identification of specific strain nodule occupancy. Field-based studies of rhizobia have shown both predominate nodulation by inoculant strains (Denton et al., 2003; Beyhaut et al., 2006) and failure of inoculant rhizobia DNA to significantly occupy nodules from sampled legumes (Malek et al., 1998; Denton et al., 2002; Seehaver, 2013). In agricultural fields, plant, microbial, and edaphic factors can influence whether rhizobia in the applied inoculant are able to sufficiently populate soil and become competitive for nodule occupancy. To better understand factors affecting rhizobia-plant dynamics in agricultural fields, a first step is to explore how rhizobia compete for nodulation, and how combinations of rhizobia affect growth and BNF efficiency (N mass per nodule mass) of legumes.

Inoculants are considered beneficial for legume growth in general, but the relative nodule occupancy and contribution to BNF by individual strains, particularly when competing against other strains, is not well understood. Our research seeks to improve our understanding of the effects of Rlv strain competition on hairy vetch growth, nodulation, and BNF with specific objectives to (1) determine if Rlv strain combinations had additive effects on BNF and nodulation of vetch based on individual strain characteristics, and (2) assess whether relative BNF capacity of rhizobia strains regulates vetch nodule occupancy. Based on the evidence, we propose that that adding less efficient rhizobia to inoculants would decrease BNF compared to inoculants with more efficient rhizobia and that changes in BNF for one inoculant compared to others would accurately reflect changes in nodule occupancy by individual rhizobia strains.
2.2 Materials and Methods

2.2.1 Rhizobia Culture Selection

Three soil isolates and one commercial strain of Rhizobium were selected from a library of strains based on symbiotic efficiency for nodulation and BNF. Rhizobia strains NCSU332 and NCSU478 were extracted from nodules of hairy vetch plants collected from sites at the Piedmont Research Station in Salisbury, North Carolina, USA (35°41'49.9"N 80°37'14.0"W). Initial observations were that plants inoculated with NCSU332 had higher than average nodule counts. NCSU478 was isolated from plants with large N to nodule mass ratios, suggesting that it had a high efficiency for BNF. Strain NCSU435 was collected from a nodule of a hairy vetch plant grown at the Cherry Research Station in Goldsboro, North Carolina, USA (35°23'28.1"N 78°01'44.0"W). Hairy vetch infected with NCSU435 were of moderate growth and nodulation. A commercial strain, C10, was isolated from hairy vetch inoculated with a commercial powder inoculant containing rhizobia for peas, lentils, and vetches.

Each rhizobia strain was initially isolated for the study by plucking nodules from hairy vetch roots and surface sterilizing them with 70% ethanol, 3% bleach, and sterile water before aseptically crushing them onto yeast-extract mannitol (YM) agar plates colored with Congo red dye (Graham, 1962). After four days of growth, colonies were taken from YM agar plates with a sterilized inoculation loop and inoculated into 1.7 mL microcentrifuge tubes containing 500 μL of tryptone yeast-extract (TY) nutrient solution for rhizobia (Graham, 1962). TY cultures were placed on a shaker for four days to allow sufficient growth of cells before being re-isolated on YM agar plates to ensure purity of a single rhizobia strain. After three isolations, 250 μL of TY culture solution with rhizobia cells was aseptically pipetted into 1.7 mL microcentrifuge tubes with 250 μL of 50% glycerol solution.
for a final concentration of 25% glycerol. Stock rhizobia cell cultures were preserved by storage at -80°C.

To establish a rhizobia cell count, cultures were removed from -80°C storage and aseptically inoculated onto individual YM agar plates. After four days of growth on the plates, single colonies from each strain were inoculated into individual 1.7 mL microcentrifuge tubes containing 700 μL of TY nutrient solution. The tubes were incubated on a shaker at room temperature for four days and allowed to reach exponential growth stage. To equalize cell concentrations, 200 μL from each cell solution was diluted to an optical density where absorbance at 600 nm wavelength (OD$_{600}$) = 0.50 ± 0.01 absorbance units. Series dilutions of $10^{-1}$ to $10^{-7}$ for the selected OD$_{600}$ cell cultures were made by mixing cell cultures with sterile water in 3 mL glass test tubes. Aliquots of 0.1 mL from each dilution were spread onto individual YM agar plates and allowed to sit in an incubator at 27°C for four days. Bacteria colonies counted on YM agar plates were used to calculate the number of colony forming units (CFU) in solution at OD$_{600}$ = 0.5 for the four selected rhizobia strains.

2.2.2 Experimental Design and Preparation

To evaluate the effects of inoculant rhizobia strain combinations on vetch BNF, treatments were established using one to four strain combinations of the selected rhizobia. There were 15 combinations of the four rhizobia strains comprising 15 different treatments to assess inoculant effects on vetch growth and nodulation. Two uninoculated control treatments included a negative control without added N and a positive control with N fertilized plants. A total of 68 plants comprised the 17 treatments, with four repetitions per treatment used in the experiment (Table 1).
Growth pots were assembled using 3 x 3 x 4 inch polycarbonate Magenta® plant culture boxes (Magenta LLC, Chicago, IL). Each unit consisted of three Magenta® boxes in which one was used as a top cover, one to hold soil, and the other as the container for the supplied nutrient solution (Figure 2.1). An approximately 1:1 volume mixture of vermiculite and sandy soil was poured into the soil compartment. A cotton rope was pulled through a hole from the soil compartment into the bottom nutrient solution container. Wicking of nutrient solution into the soil compartment maintained moisture and nutrient supply to avoid disturbing the soil surface from direct watering. N-free nutrient solution containing 1 mM CaSO$_4$, 0.5 mM KH$_2$PO$_4$, 10 µM ferric citrate, 0.25 mM MgSO$_4$, 0.25 mM K$_2$SO$_4$, 1 µM MnSO$_4$, 2 µM H$_3$BO$_3$, 0.5 µM ZnSO$_4$, 0.2 µM CuSO$_4$, 0.1 µM CoSO$_4$, and 0.1 µM Na$_2$MoO$_4$ was prepared as previously described (Broughton and Dilworth, 1971). N-free nutrient solution was supplied to negative-N plants and inoculated plants whereas a similar nutrient solution containing 2.5 mM NH$_4$NO$_3$ was supplied to positive-N plants. All growth units were sealed and sterilized in an autoclave at 120°C for two hours prior to planting.

Vetch seeds were surface sterilized in 70% ethanol for 30 seconds and transferred to 4% bleach to soak for five minutes. Three seeds were placed aseptically into each growth unit, and all growth units were subsequently transported to an 8 x 4 x 7 foot controlled growth chamber at the North Carolina State University Phytotron. The walk-in growth chamber provided nine hours of fluorescent light at a temperature of 22°C and no light at 18°C for 15 hours. The average photosynthetic photon flux density was 620 µmol·m$^{-2}$·s$^{-1}$, the relative humidity was maintained near 70%, and CO$_2$ concentration varied between 300-400 ppm. Units were arranged in a randomized complete block design and rotated around the chamber every four days. After four days, when the
vetch seeds sprouted, plants were thinned to the single largest sprout. Sterilized trellises were placed in each unit to provide support for growing vetch shoots.

Rhizobia cultures in TY nutrient solution were incubated for four days and diluted to OD$_{600} = 0.5$. Culture solutions were then diluted to a final concentration of $10^7$ CFU mL$^{-1}$ based on plated cell counts determined for earlier dilutions. The cultures were mixed in 45 mL tubes so that an equal number of CFU from each strain (as calculated earlier) was placed into single and combined inoculants (Table 2.1). Liquid inoculant solutions were pipetted in 2 mL aliquots around the base of the remaining seedlings in their respective growth units.

2.2.3 Plant Analysis

Inoculated vetch plants were allowed to grow 46 days before extraction for N and nodule analyses. Shoots were cut from roots slightly above the soil surface in the pots. Each of the 68 shoots was enclosed in individual paper bags and placed in a forced air oven at 60°C for 24 hours. After the dry mass of each shoot was measured and recorded, plant shoots were homogenized using a Cyclotec 1093 Sample Mill (Foss Tecater, Höganäs, Sweden) and stored in polyethylene vials until further analysis.

Analysis of N concentration of plant tissue (10 mg) was conducted by the North Carolina State University Environmental and Agricultural Testing Service (EATS) Lab using a Perkin Elmer PE2400 CHNS elemental analyzer (Perkin Elmer, Waltham, MA). The N concentration of representative tissue samples was used to represent the total N concentration of whole shoots. Total shoot mass and N concentration of each treatment was then used to calculate the total N mass in each shoot. The average total N mass of negative-N controls was used to calculate the
average N content of vetch seeds. BNF was determined by subtracting the average N content of negative-N plants from the calculated amount of N of individual inoculated plants.

Roots were stored at 4°C briefly after extraction until nodules could be removed. Nodules were quantified before being placed in desiccator vials to dehydrate. After one week of drying, the total mass of dry root nodules for each plant was measured, and nodules were stored in polyethylene vials until further analysis.

2.2.4 DNA Extraction

Pure cultures of Rhizobium leguminosarum (RLv) strains C10, NCSU332, NCSU435, and NCSU478 were inoculated into TY nutrient solution and incubated at room temperature for four days. Cells in TY were pelleted and the supernatant discarded. Suspension and pelleting of cells occurred twice in 70% ethanol before the ethanol supernatant was discarded. Then, 200 μL of sterile distilled water was used to suspend cells in solution.

A total of 270 nodules from 45 plants (Table 2.2) were selected for DNA extraction. To capture potential variability in plants inoculated with more than one strain, the number of nodules selected at random from each plant was determined by the number of inoculant strains used. From each of 12 plants inoculated with one strain, three nodules were selected. From each of 18 plants inoculated with two strains, six nodules were selected. From each of 12 plants inoculated with three strains, eight nodules were selected. From each of three plants inoculated with four strains, ten nodules were selected. The number of nodules occupied by a strain in each plant was converted into a percentage per treatment to represent relative nodule occupancy.
Prior to DNA extraction, desiccated nodules were imbibed in sterile water for approximately 18 hours (overnight). To kill surface contaminants, nodules were placed in 3% bleach for five minutes and rinsed in sterile water for one minute. After sterilization, each nodule was transferred to a 2.0 mL lysis tube. Genomic DNA was extracted from nodule tissue following a modified protocol of the FastSpin DNA Kit (MP Biomedicals, Santa Ana, CA). A lysis tube with one nodule was filled with 800 µL of CLS-TC (containing sodium-dodecyl-sulfate) and 200 µL of protein precipitation solution (PPS). A FastPrep® FP120-A115 homogenizer (Qbiogene, Inc., Carlsbad, CA) set to speed 6.0 for 45 seconds was used to disintegrate nodule tissue. Each tube was centrifuged at 14,000 × g for 7.5 minutes. 650 µL of supernatant from each lysis tube was transferred to a new 2.0 mL tube and 650 µL of DNA binding matrix was added. The tube with supernatant and binding matrix was agitated at low speed for 5 minutes to sufficiently mix DNA with binding matrix. The solution was transferred to a 2.0 mL tube with a catch filter attached and centrifuged to remove liquid from DNA binding matrix. 500 µL of SEWS-M (containing 90% ethanol) suspended the DNA binding matrix above the catch filter to precipitate out DNA. The catch tube was twice centrifuged for one minute and emptied to remove excess SEWS-M. Binding matrix in the catch tube was suspended in 100 µL of DNase-free water at 55°C for five minutes. Centrifugation of the water into a clean 2.0 mL tube finally isolated genomic DNA from nodule and rhizobia tissue. DNA concentration and purity was measured using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). All samples were stored at -20°C until further analysis.

2.2.5 nodC PCR

The presence of Rlv DNA in nodule DNA extracts was confirmed by amplification of nodC gene fragments. Primer sequences nodCfor540 and nodCrev1160 (Sarita et al., 2005) were used
because of their specificity to rhizobia species (Table 2.3). Information from nodC gene sequences identified in Rhizobase (Kazuka DNA Research Institute, 2011) and the *Rhizobium leguminosarum* genome project (Young et al., 2006) was used to compare nodC sequences from strains.

The amplification solution used for nodC PCR contained 12 µL of PCR water, 8 µL of 2X Apex® Red Taq (Genesee Scientific, San Diego, CA), 0.5 µL of each primer (final concentrations of 0.45 µM), and 1 µL of template DNA. The protocol for nodC amplification was optimized to an amplification profile: initial denaturation at 94°C for three minutes; 33 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, extension at 72°C for one minute; and a final extension at 72°C for five minutes. nodC PCR products were transferred to a 1.5% agarose gel made with Tris/borate/EDTA (TBE) solution and submerged in 1X TBE in distilled water. Electrophoresis was conducted at 105 V for two hours. All nodC PCR products were visualized under UV light using a Syngene Genegenius Super 12 transilluminator (Synoptics Ltd., Cambridge, UK) and photographed using Syngene 6.08 software (Synoptics Ltd., Cambridge, UK).

2.2.6 BOX-A1R PCR

BOX-A1R primer, used to generate DNA fingerprints of bacterial DNA, profiled individual rhizobia strains and DNA extracted from nodules. The primer was designed to target the 16S–23S region of bacterial DNA and can generate strain specific DNA fingerprints after PCR amplification (Koeuth et al., 1995). The amplification solution for BOX-A1R fragments contained 14 µL PCR water, 8 µL of 2X Apex Red Taq, 1 µL of 20 µM BOX-A1R primer (final concentration 0.83 µM) and 1 µL of template DNA. A PCR protocol was arranged as: Initial denaturation at 94°C for three minutes; 35 cycles of denaturating at 94°C for one minute, annealing at 55°C for one minute, extension at 72°C for one minute; and a final extension at 72°C for five minutes. PCR products from BOX-A1R fragment
amplification were transferred to a 3% agarose gel in 1X TBE. Electrophoresis occurred at 85V for 18 hours. PCR amplicons in the gel were visualized under UV light using a Syngene Genegenius Super 12 and photographed using Syngene 6.08 software.

All BOX-A1R DNA fingerprints were entered into GelCompar II (Applied Maths NV) to be analyzed for similarity. Similarity between rhizobia BOX-A1R DNA fingerprints was determined using the Dice similarity coefficient with 1% tolerance and neighbor joining cluster analysis. Confirmation of strain fingerprints in nodules was used to calculate the proportion of nodules occupied by initial inoculant strains as well as outliers that may not align with pure strain DNA fingerprints.

Nodule DNA extract was diluted with PCR water to reduce concentrations of amplification inhibitors. Comparisons were made between initial and diluted extracts to ensure that rhizobia DNA concentrations were sufficient and DNA fingerprint clarity was improved (Santasup, 2000).

2.2.7 Statistical Analysis

Differences in vetch growth between treatments were evaluated using an analysis of variance (ANOVA) procedure and Statistical Analysis System (SAS) software version 9.4 (SAS Institute, Inc., Cary, NC). Least significant differences (LSD) from the F-test method (p = 0.05) were used to compare treatment averages of shoot mass, shoot N concentration, total shoot N, nodule count, nodule mass, and BNF efficiency (N mass per total nodule mass). Correlations between vetch responses were evaluated using Microsoft Office Excel linear regression analysis package (Microsoft Corporation, Redmond, WA, USA).
2.3 Results

2.3.1 Inoculant Concentration

Cultures from the selected rhizobia strains measured at OD₆₀₀ = 0.5 had different cell counts in TY nutrient solution. C10, NCSU332, NCSU435, and NCSU478 contained 6.5 x 10⁷, 6.8 x 10⁷, 7.9 x 10⁷, and 8.6 x 10⁷ CFU mL⁻¹, respectively. Cell counts are comparable to those seen in other rhizobia studies with similar incubation time (Yahalom et al., 1987; Malek et al., 1998). Each cell culture was diluted to 1 x 10⁷ CFU mL⁻¹ to facilitate application in multi-strain trials when preparing mixed inoculants.

2.3.2 Plant Growth

Shoots from inoculated plants were visibly different in size after 46 days in the growth chamber (Figure 2.1). As expected, negative-N controls and positive-N controls were the most visibly different in growth compared to other treatments. Negative-N controls accumulated shoot mass ranging 0.12–0.15 g plant⁻¹, with an average of 0.14 g plant⁻¹ (Table 2.4). Because no rhizobia inoculant was used and little N is assumed to be contained in the growth medium, the plant N in the negative-N controls was assumed to be derived almost entirely from seed storage. In contrast, positive-N controls accumulated shoot mass ranging 1.71–2.03 g plant⁻¹, and averaged 1.90 g plant⁻¹, or nearly 14 times more mass than negative-N controls.

All inoculated vetch accumulated more shoot mass than negative-N controls on average, but less shoot mass than positive-N controls. Average shoot mass among inoculated treatments ranged 0.56–1.57 g. Single-strain inoculants NCSU332 and NCSU435 averaged the least shoot mass among inoculated treatments with 0.63 g and 0.56 g respectively (Table 4). Single-strain inoculation by C10
and NCSU478 produced greater shoot mass, with 1.07 g and 0.95 g average shoot mass respectively, but these values were not significantly different from NCSU332 and NCSU435. In all but two cases, average shoot mass exceeded 1.0 g for trials with multiple strains, which was more than most of the individual strain shoot masses. Additionally, four treatments were statistically similar to positive-N vetch in terms of shoot mass, and all four of those treatments were multi-strain inoculants.

2.3.3 Biological Nitrogen Fixation

Shoot tissue N concentration and total N masses are shown in table 2.4. Shoot N concentration ranged from 4.37 to 5.32% and was within the range expected for N in vetch tissue (Degregorio et al., 1995; Lawson et al., 2012; Mothapo et al., 2013). The fact that all individual plant shoots exceeded 4% N concentration (see appendix) is noteworthy because yields with 4% N or greater surpass the typically reported N concentrations of other cover crop legumes (Hargrove, 1986; Parr et al., 2011).

The treatment with the lowest shoot N concentration was the vetch that received NH₄NO₃ (Table 4). Although this may seem counterintuitive, similar results for legume growth were seen by Lagurre et al. (2007), who compared Rlv inoculated pea plants (Pisium sativum) to N-fertilized plants. They rationalized the observation by noting oversupply of the limiting nutrient may allow N-fertilized plants to accumulate more of the other nutrients required for growth, which subsequently diluted the N concentration in plant tissues. However, in terms of total mass of N per shoot, the negative-N plants had the least total N and shoot mass whereas N-fertilized vetch contained the most N and shoot mass. For vetch plants inoculated with one or more Rlv strains, 11 of the 15 treatments were statistically similar in average total N to the fertilized vetch (p < 0.05) (Table 4). Compared to N-fertilized plants, the greatest N content in inoculated trials reached 93% total N of
vetch supplied with NH₄NO₃ and the least N yield was 31%. Average N content shoot⁻¹ was greatest in plants inoculated with NCSU478. There were no statistically significant differences or trends observed among any of the single or multi-strain inoculants for N concentration in vetch shoot tissue.

Negative-N plants had senescent leaves and stunted growth, and were believed to have utilized the majority of their available seed N content. Based on this assumption and total N content of controls, inoculated trials were corrected by 6.3 mg N plant⁻¹ to account for seed storage of N. Inoculated treatments generated 19.6–70.9 mg N from BNF (Table 2.5). Single-strain inoculants C10, NCSU332, NCSU435, and NCSU478 produced 43.4, 23.4, 19.6, and 44.2 mg N, respectively. NCSU478, a field isolate, produced the most total N assimilated into vetch on average and the commercial inoculant, C10, fixed nearly twice as much N as two of the three field isolates on average, but results were not statistically significant.

Although differences among single strain and multiple strain inoculants were not statistically significant, strong trends were observed. In four of six cases, treatments with two inoculant strains averaged more total BNF than the more effective single-strain inoculants, C10 and NCSU478 (Table 2.5). In all 6 cases, treatments with two inoculant strains averaged more total BNF than the strains that produced less BNF individually, NCSU332 and NCSU435. In addition, each two-strain inoculant treatment containing C10 increased in total BNF over the C10 inoculant alone. In contrast, the lower performing strains combined with NCSU478 in inoculants resulted in a decrease in total BNF compared to NCSU478 alone. The combination of NCSU332 and NCSU435 produced 49.0 mg N, which was more BNF than the two strains individually combined with either C10 or NCSU478. It
should be noted that most of these differences are small, and none of the trials containing two inoculants produced statistically different average BNF from one another (p < 0.05).

In trials with three and four inoculants, BNF ranged 36.3–55.2 mg N, with three of five combinations exceeding 50 mg N. In general, average BNF with three and four-strain inoculants, 48.0 mg N, was greater than the average of trials with one strain, 37.7 mg N, and comparable to two strains, 48.5 mg N. Thus, BNF in multi-strain inoculation was generally improved over trials with one strain.

2.3.4 Nodulation and BNF efficiency

Nodule number and mass varied greatly among treatments (Table 2.5). The strain producing the fewest nodules on average was C10, with an average of 31 nodules weighing 29.7 mg total. In contrast, NCSU478 produced 62 nodules weighing 46.1 mg total. Although C10 was the least frequent nodulator, it produced more nodule mass than both NCSU332 and NCSU435. Differences among single and multiple strains were not statistically consistent.

Treatments with two inoculant strains ranged 31–70 nodules and 22.9–52.0 mg nodule mass. There was no statistical difference between nodule count or nodule mass in treatments with one inoculant and those with multiple strains (p < 0.05), which was likely due to the large range in nodulation within treatments causing large variance. For example, nodule number for plants inoculated with C10 and NCSU478 combined ranged from 42 to 109 nodules.

Among all treatments, average total nodule mass correlated (p < 0.05) more strongly with total BNF than average nodule number (Figure 2.2a and 2.2b). Thus, average total nodule mass and total BNF were used to calculate BNF efficiency as BNF per nodule mass for each inoculated trial.
For single-strain treatments, strain C10 had the largest BNF efficiency at 1.5 mg N mg nodule\(^{-1}\). Of the single-strain inoculants, NCSU435 was the least efficient N fixer and C10 was the most efficient. Although BNF efficiency was not statistically different between the individual strains the data showed that strain C10 was the most efficient on average (Table 5).

BNF efficiency of multi-strain inoculants was generally greater compared to single-strain inoculants. Multi-strain inoculants ranged in BNF efficiency from 0.93 to 2.25 mg N mg nodule\(^{-1}\). In all but one case, multi-strain inoculants resulted in a BNF efficiency exceeding that of treatments with single-strain inoculants but differences were not statistically significant at \((p = 0.05)\). The inoculant combining strains C10 and NCSU332 had significantly greater BNF efficiency than three single-strain inoculants (Table 2.5), but there was no observable pattern in BNF efficiency among multi-strain inoculant treatments.

### 2.3.5 Nodule Occupancy

Nodule occupancy was determined by PCR amplification of DNA with BOX-A1R PCR primer followed by imaging of electrophoretically separated fragments of DNA from the 16S–23S region. All strains produced distinctive DNA fingerprints (Figure 2.3), which are also clearly indefinable in DNA extracted from harvested nodules of inoculated vetch (Figure 2.4). In all single-strain trials, the applied Rlv strain was exclusively recovered in all sampled nodules (Table 2.6), validating the approach for determining nodule occupancy.

DNA fingerprinting was utilized to determine the fraction of nodules occupied by strains on inoculated vetch roots. Although nodule occupancy of combined inoculants was expected to trend with either average BNF efficiency or nodule counts observed from single-strain trials (Vlassak and
Vanderleyden, 1997), the data suggest competition between strains may be more complicated than predicted. In competitive trials with strains C10 and NCSU332, C10 was found in 67% of analyzed nodules, which was twice as frequent as NCSU332, a more prolific nodulator in single-strain trials (Table 2.6). In contrast, C10 occupied only 44% of nodules in trials that also contained NCSU435 or NCSU478. Strain NCSU478 generated the most nodules as a single-strain inoculant and was expected to occupy most nodules from combined inoculants, but it only occupied a majority of nodules when combined with C10. When combined with strains NCSU332 or NCSU435, it only occupied 33% and 17%, respectively.

To compare treatments with different numbers of inoculants, the fraction of nodules was normalized by the number of strains in each treatment (Figure 2.5), with a value of 1 representing expected occupancy assuming equal competitiveness. Across all treatments, the most frequent nodule occupant in direct competition with other Rlv strains was NCSU435, whereas the others had lower occupancies (normalized occupancy < 1). In fact, NCSU435 occupied more nodules than any other strain and had occupancy greater than 50% in two of three triple-strain treatments including it (Table 2.6). Surprisingly, NCSU478 was the least frequent occupant in treatments containing two strains despite producing the most nodules as a single-strain inoculant. In fact, nodule occupancy by NCSU478 did not exceed 13% for any treatment with three or more strains. In general, C10 and NCSU332 were intermediate in their nodule occupancy, and similar in relative nodule occupancy in multi-strain treatments.

Some nodule analyses resulted in failed BOX-A1R PCR amplification (no amplification) or BOX-A1R DNA fingerprints that did not match the pure strain DNA fingerprints. Nodules with no amplification after repeated attempts, which are interpreted as nodules containing insufficient
amounts of rhizobia DNA (Banba et al., 2001; Van de Velde et al., 2006), were most common in trials with three or more inoculants. No amplification can result when symbiosis is terminated by the host plant and nodules contain insufficient amounts of rhizobia DNA (Oono et al., 2011). Fingerprints that do not match inoculated strains may indicate the presence of contaminant rhizobia strain (e.g., a strain that was not intentionally introduced) or possibly nodules containing more than one Rlv strain (Bogino et al., 2011), which would result in different DNA amplification compared to pure strain DNA amplification.

2.4 Discussion

2.4.1 Strain-related differences in BNF

Biomass accumulation in a plant is directly related to N supply. The average plant mass of each treatment was strongly correlated with total N in shoots (Figure 2.6), and therefore inoculant strains producing the most BNF produce the most vetch biomass. Vetch response to inoculation by individual Rlv strains is potentially characteristic of the inoculant strain used (Fesenko et al., 1995; Thrall et al., 2000) because of the observed trends in BNF efficiency. Differences in BNF have been attributed to strain genotypes that may provide resistance to restrictive environmental conditions, which could be related to native environments (Elsheikh and Wood, 1995). In our study, the four selected strains were from three sources: two locations in North Carolina, and one commercial peat inoculant for vetch. BNF efficiency was greatest from the commercial peat inoculant strain, C10, and measured 50% more BNF than the best performing field isolate, NCSU478, but total BNF was statistically similar between the two strains. The two field isolates from Salisbury, NC were the second and third most efficient strains, and the Goldsboro, NC strain had the lowest BNF efficiency. Although characteristics of the location from which C10 was isolated are unknown, it is possible that
they are unlike those from which our isolates were obtained. Soil is sandier in Goldsboro compared to Salisbury, but the two areas are climatically similar. Differences in geographic origin may have contributed to variability between rhizobia strain BNF with vetch, but they were not strong enough to produce significant differences in BNF produced by the selected strains. Other studies have found correlations between geographic variation in inoculant rhizobia symbiosis genes and larger differences in BNF for the same legume host (Rincón-Rosales et al., 2009).

Differences in BNF by rhizobia strains have also been attributed to variance in relevant symbiosis genes (Devine and Kuykendall, 1996). The selected rhizobia differed in their nodC genes by 3–5.5% (data not shown), which was less than the genetic difference of rhizobia reported elsewhere (Laguerre et al., 2003; Granada et al., 2014), but this difference itself is not a diagnostic characteristic of Rlv performance for BNF. Although comparative genetic studies have been useful in determining the diversity of rhizobia communities (Dresler-Nurmi et al., 2009; Rogel et al., 2011), specific genetic parameters to determine strain-level differences in symbiosis or BNF capacity have not yet been identified.

2.4.2 Relationship between Nodule count, mass, and BNF

Evolutionary theory of mutualistic symbiosis suggests that the most efficient rhizobia more frequently nodulate legume roots because they have the greatest potential to increase plant growth (Archetti et al., 2011; Friesen, 2012). In our study, a correlation was observed between nodule count and BNF (Figure 2.2a). However, a trend between nodule number and strain performance was not observed within averages of individual Rlv strains, which is consistent with the observation that rhizobia most efficient at fixing N do not always produce the most nodules (Hafeez et al., 2001; Oono et al., 2009). For example, in single-strain trials, strain C10 had the greatest BNF efficiency, but
produced the fewest nodules. Limits on nodulation have been observed in legumes receiving sufficient N supply (Ferguson et al., 2010), but it is doubtful that fewer nodules were produced by C10 because of that reason. Also, twice as much N was yielded from strain C10 inoculation than strains NCSU332 and NCSU435, even though nodule number was 50% greater from the two less efficient strains. Although increased nodulation by NCSU478 resulted in more BNF, observations from the NCSU332 and NCSU435 contradict suggestions that less efficient rhizobia nodulate more often in order to fix more N for the host legume (Oono et al., 2009).

Nodule number indicated successful nodulation events, but continued growth of rhizobia in nodules, indicated by nodule mass, was better correlated to total BNF. Although there were no strain specific differences in nodule mass, on a per mass basis strain C10 fixed more N through symbiosis than the others despite producing fewer nodules. Symbiotic efficiencies of strains has been measured in previous research suggesting favorable allocation of resources to growth of more efficient nodulating rhizobia (Kiers et al., 2006; Sachs et al., 2010b). Our results are consistent with the idea that vetch may have been optimizing growth of C10, the most efficient strain in our trials, more than the other strains. Consequently, a less efficient strain could produce significantly more nodules than a more efficient strain and still not produce as much BNF through symbiosis (Friesen, 2012). Because nodule mass was more closely related to BNF than nodule number, it was appropriate to evaluate BNF efficiency on a per total nodule mass basis.

2.4.3 Nodule Occupancy

In general, nodule occupancy by combined inoculants was not favorable to the strain producing the most nodules or the strain with the greatest BNF efficiency as a single-strain inoculant. Instead, NCSU435 occupied more nodules than the other strains even though it did not
produce significantly more BNF for vetch growth. In trials with two strains, predominant occupancy (>80% of nodules) by a specific rhizobia strain is commonly observed (Denton et al., 2002), but preferential nodule occupancy for a single rhizobia genotype in entire communities is less common. Nodule occupancy is rarely greater than 30% when several strains are competing for nodulation (Ballard et al., 2004), and in some studies inoculant rhizobia are recovered from significantly fewer nodules than resident rhizobia when applied as inoculants in the field (Spriggs and Dakora, 2007; Seehaver, 2013). Overall, our results do not support the idea that symbiotic efficiency of individual strains is a controlling factor for nodule occupancy or that vetch is preferentially selective for Rlv strains producing the most BNF.

Differences between BNF efficiency in single-strain trials and nodule occupancy in multi-strain trials confirmed that single-strain characteristics were not necessarily representative of competitive ability (Friesen, 2012). There is considerable interest in studying potential factors controlling nodule occupancy (Vlassak and Vanderleyden, 1997) in order to develop inoculants that are more successful at competing with resident rhizobia. Success of symbiosis can vary depending on nodulation signals exchanged between the host legume and its many potential rhizobia partners (Gubry-Rangin et al., 2010), but contradictory observations have been made about the ability for legumes to distinguish between rhizobia. Some researchers refer to recognition of specific nodulation signals by legumes (Deakin and Broughton, 2009; Sachs et al., 2010) whereas others postulate that frequency of nodulation may be characteristic of entire rhizobia populations rather than individual strains (Denison, 2000). In the latter scenario, if nodulation of vetch is regulated by the sum of all nodulation signals rather than signals from individual strains, opportunities to nodulate roots may be available to all compatible rhizobia in the rhizosphere. The release of nod
factors from any of the strains in soil would therefore benefit the entire community rather than whichever strains stimulate roots the most. This idea is thus compatible with our observation that, although no statistically significant strain-dependent behavior was observed in multi-strain treatments, nodule count and BNF generally increased for multi-strain inoculants compared to single-strain inoculant treatments.

2.4.4 Predicted BNF vs Actual BNF

Assessing ineffective and effective rhizobia occupying vetch nodules helped determine the overall effect of strain combinations on BNF. Nodule number, nodule occupancy, and BNF efficiency per nodule from the nodule occupants could be used to calculate theoretical BNF of inoculated vetch with an additive model from single-strain trials:

\[ \text{Predicted BNF plant}^{-1} = \sum_{i=1}^{n} \text{Number of nodules} \times \text{Strain}_i \text{nodule occupancy} \times \text{Strain}_i \text{BNF efficiency} \]

Plotting the theoretical BNF against the actual BNF allows for determination of the deviation of BNF from additive model of efficiency. The resulting graph revealed significant relationships (p < 0.05) between actual and predicted BNF for individual plants of both single-strain and multi-strain trials (Figure 2.7), but not averages of experimental treatments (not shown). This emphasizes the relationship between BNF and occupancy, and also illustrates the variability of BNF and nodulation within treatments. Predicted BNF from single-strain inoculants correlated with actual BNF (r^2 = 0.69), but limitations of the quantitative predictive ability of this approach were highlighted by the deviation of the best-fit line from the expected value of unity.

A significant relationship was also observed between predicted and actual BNF in multi-strain trials. In a truly additive system, there would be a one-to-one correlation between the two
values. However, the relationship exhibits a line with non-unity slope and a higher intercept than would be predicted, suggesting a higher minimum BNF for combined inoculants than would be predicted based on observed single-strain inoculant efficiencies. Decreased predictability of multi-strain inoculant BNF was expected because of the complicated interactions between vetch roots and Rlv strains (Dowling and Broughton, 1986).

In addition, inspection of figure 2.7 shows that actual BNF from plants in multi-strain trials tends to exceed those from single-strain trials despite single-strain trials having more predicted BNF. More BNF from multi-strain inoculation relative to single-strain inoculation indicates that BNF efficiency was not a combined efficiency of more effective and less effective strains. It was expected that the less effective Rlv strains would decrease overall BNF when combined with more effective strains and that total BNF would be limited by the most efficient strain. Instead, several strain combinations fixed more N and had better BNF efficiency than the individual strains used as inoculants. The comparison between predicted and actual BNF indicated that BNF can be improved regardless of the strains used in combined inoculants.

2.5 Conclusions

Because vetch is a legume, N can be assimilated from soil and through BNF with its symbiotic partner, Rlv. Vetch plants were grown without N and inoculated with rhizobia to assess how BNF and nodulation of roots was affected by different combinations of Rlv strains. As expected, vetch growth increased with increasing N content, and the range of N assimilation in the inoculated plants was between that of uninoculated negative-N and positive-N plants. Although nodulation and total nodule mass of single-strain inoculants was variable, on average the commercial inoculant strain trended higher than the others in BNF efficiency which indicated a potential strain effect on N
assimilation from BNF in the rhizobium-legume symbiosis. Surprisingly, the strain most efficient at BNF was not the strain producing the most nodules, as would be expected for evolutionary preference. The strains were combined in inoculants to assess how single strains competed for symbiosis with vetch. Investigation of nodule occupancy with PCR DNA fingerprinting indicated that neither relative nodulation nor relative BNF efficiency were indicative of nodulation by specific rhizobia strains in multi-strain inoculants.

Despite individual strain characteristics not being predictors of relative nodule occupancy, the collected data was used to predict total BNF from combined inoculants based on results from single-strain trials. Predicted BNF calculated from nodule number, nodule occupancy by the four strains, and strain BNF efficiency was a good relative predictor of total BNF from strain treatments but not an absolute predictor. In general, BNF was increased in multi-strain treatments, but this was not attributable to the action of any one strain. Our results suggest that a diversity of rhizobia nodulating vetch may be more important for improving BNF than the BNF capacity of a single strain (Duodu et al., 1999; Denison and Toby Kiers, 2004). Switching inoculant strains did not have a significant effect on BNF, but including more rhizobia to improve symbiotic efficiency of an inoculant was generally beneficial. This observation is consistent with previous studies observing that inoculation either maintained or improved BNF in vetch and other legume crops (McKenzie et al., 2001; Hungria et al., 2003; Kaschuk et al., 2010) even when resident populations dominated nodule occupancy.

**References cited**


Rincón-Rosales, R., L. Lloret, E. Ponce, and E. Martinez-Romero. 2009. Rhizobia with different symbiotic efficiencies nodulate Acaciella angustissima in Mexico, including Sinorhizobium chiapanecum sp. nov. which has common symbiotic genes with Sinorhizobium mexicanum. FEMS Microbiol. Ecol. 67(1): 103–117.


Table 2.1  Treatments for Rlv inoculation of vetch grown within a controlled growth chamber

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reps</th>
<th>C10 volume mL</th>
<th>NCSU 332 volume mL</th>
<th>NCSU 435 volume mL</th>
<th>NCSU 478 volume mL</th>
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<td>0</td>
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<td>0</td>
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<td>0.67</td>
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</tr>
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All solutions contained $10^7$ CFU mL$^{-1}$ as measured by OD$_{600}$=0.5 absorbance units
Figure 2.1 Photographs taken to compare growth of negative-N controls, inoculated vetch, and positive-N controls. a) Negative-N control vetch plant b) Vetch plant inoculated with strains C10 and NCSU332 c) Vetch plant inoculated with C10 and NCSU478 d) Positive-N control vetch plant
**Table 2.2** Number of plants and nodules selected for each treatment to evaluate nodule occupancy

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of plants</th>
<th>Nodules per plant</th>
<th>Total nodules</th>
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<td>9</td>
</tr>
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<td>9</td>
</tr>
<tr>
<td>NCSU478</td>
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<td>3</td>
<td>9</td>
</tr>
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<td>18</td>
</tr>
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<td>18</td>
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<td>18</td>
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<td>18</td>
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<td>NCSU 332/478</td>
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<td>18</td>
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<td>6</td>
<td>18</td>
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<td>24</td>
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<td>24</td>
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<td>8</td>
<td>24</td>
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<td>30</td>
</tr>
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Table 2.3 Primers used for PCR amplification of rhizobia DNA extracted from nodules and pure cultures

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<th>Molecular weight</th>
<th>Source</th>
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<td>TGA TYG AYA TGG ART AYT GGC T</td>
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<td>6805.0</td>
<td>Sarita et al., 2006</td>
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<td>64.5°C</td>
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<td>BOX-A1R</td>
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<td>Koeuth et al., 1995</td>
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Table 2.4 Averages for shoot mass, N concentration, and total N of vetch from different treatments

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<th>Shoot N concentration</th>
<th>Shoot total N</th>
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Values presented are the average of 4 replicates.
Significance determined using Tukey paired LSD (p = 0.05).
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<th>Nodule count</th>
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<td>mg</td>
<td>mg nodule⁻¹</td>
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* BNF calculated by adjusting total plant N (mg) by negative-N control treatment average of 0.14 g of total shoot N. Values presented are representative of four replicates. Significance was determined using Tukey paired LSD (p = 0.05).
Figure 2.2 Correlation between shoot BNF and nodulation of individual inoculated vetch plants. a) Nodule number and b) Total dry nodule mass
Figure 2.3. Electrophoresis gel image of BOX-A1R PCR amplified DNA from pure cultures of the selected RLv strains. Lane 1) DNA Ladder 2) C10 3) NCSU332 4) NCSU435 5) NCSU478
Figure 2.4. Electrophoresis gel image of PCR amplified DNA using BOX-A1R primer on vetch nodule extracts. Lanes 1 and 25 contain standard DNA ladder. Lanes 2-24 contain DNA from strains C10, NCSU332, NCSU435, and NCSU478 as labeled above.
Table 2.6 Nodule occupancy of vetch roots as determined by BOX-A1R PCR DNA fingerprints from vetch nodule extracts

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<th>NCSU 435</th>
<th>NCSU 478</th>
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* Nodule DNA that generated a fingerprint different from inoculant strains.
** Strains found outside of an intended treatment possibly due to growth chamber conditions.

Occupancy expressed as the percentage of selected nodules from the treatment in which the strain DNA was found.
Figure 2.5 Normalized distribution of nodule occupancy between the four Rlv strains used as inoculants. A value of 1.0 refers to equal nodule occupancy, below 1.0 is less than equal occupancy, and above 1.0 is greater than equal occupancy. Unidentified and uninoculated nodules are not included in values.
Figure 2.6 Correlation between dry mass accumulation and shoot N determined by plant tissue analysis for each vetch plant

\[ R^2 = 0.9716 \]

\( n = 68 \)
Figure 2.7 Actual vs predicted BNF calculated based on nodule occupancy observed in inoculated plants and BNF efficiency of each rhizobia strain. Trend was statistically significant as determined by Pearson correlation coefficient. One statistical outlier was removed from analysis.
Chapter 3: Summary and Conclusions

3.1 Major findings

Although rhizobia strains may show individual BNF efficiency with vetch, in practical applications they are often competing against many other strains for occupancy of legume nodules where individual characteristics may not stand out. The biggest issues with assessing inoculant effectiveness arrived while comparing relative BNF efficiency and nodulation to individual strain nodule occupancy. Trends observed in nodule occupancy did not follow trends in nodulation and BNF from single-strain inoculants. It was expected that nodulation by Rlv strains with greater BNF efficiency would be preferred by vetch and therefore result in preferential nodule occupancy from more efficient strains. However, neither single-strain nodulation nor multi-strain nodule occupancy followed that expectation. As a result, capacity for BNF was not linked to nodulation or nodule occupancy of vetch roots when Rlv strains are in competition.

The results showed that strain combinations ultimately provided the best benefit to vetch growth. Studies of rhizobia inoculants typically use preliminary results from single-strain inoculation to evaluate strains on potential for BNF and increased yield (Ballard et al., 2004), but those results may not transfer to competitive situations when multiple strains are competing for nodule occupancy of the legume crop (Spriggs and Dakora, 2007). In the strain combinations used, both less efficient and more efficient rhizobia tended to improve growth and the overall goal of stimulating vetch roots to produce more BNF was achieved. Combined inoculant BNF may not be accurately predicted based on individual strain characteristics, but variability can be accounted for in theoretical models to assess competitive behavior (Fujita et al., 2014). Despite the observed variability, 11 of 15 inoculant treatments averaged total BNF comparable to the positive-N control.
vetch plants, which indicates that the rhizobium-legume symbiosis between hairy vetch and Rlv is highly effective for BNF.

3.2 Implications of findings

Inoculating to improve rhizobia community structure with combinations of several strains is likely to improve BNF and nodulation of hairy vetch. If nodulation and nodule occupancy are not controlled specifically by vetch then it is best to stimulate roots with as many different nodulation signaling compounds as possible to increase the chance of nodulation by all compatible rhizobia strains in the rhizosphere. For now, the focus of legume inoculation by rhizobia is better aimed at improving general rhizobia community fitness and N yields from BNF.

The benefit of BNF from the rhizobium-legume symbiosis was ultimately proven by the relative amount of BNF produced in most of the inoculated vetch compared to N-fertilized controls. Planting vetch can improve N fertility and reduce fertilizer needs because legumes contribute N to soil with little energy input. It is already known that vetch plants have a greater N concentration than many other legume cover crop species that utilize BNF, and therefore the appeal of inoculating soil with rhizobia for vetch cover crop is large. Vetch used as a cover crop is likely to produce considerable amounts of N for successive crops while also providing other cover crop benefits like soil stabilization and weed suppression. By adding more compatible rhizobia to soils, the benefits of vetch cover crop would be improved or sustained with combined community fitness of nodulating rhizobia. It is promising to know that vetch has great potential to improve soil N fertility with any combination of Rlv strains occupying nodules. Producers would likely be comfortable knowing that increased BNF is often expected when the rhizobia community grows for an inoculated vetch cover crop.
3.3 Recommendations for future research

The lack of correlation between nodulation and nodule occupancy in the rhizobium-legume symbiosis continues to complicate assessments of the relative efficacy of formulated inoculants containing highly effective rhizobia that may substantially improve BNF in legume cover crops. If rhizobia do not individually stimulate legume roots to advantageously nodulate and increase BNF, continuing to select rhizobia strains on the basis of observed nodulation and BNF efficiency would be questionable. Conditions that may allow competitive behavior to be replicated may need to be evaluated for each individual strain. Perhaps different techniques are needed to evaluate absolute competitiveness of inoculants. More advanced analyses of the rhizobia genome are likely to elucidate how the rhizobium-legume symbiosis is affected by genetic variation.

If rhizobia strains will be evaluated on their ability to compete, appropriate sampling from roots will need to account for the amount of expected competition. Legume roots sometimes have over 150 nodules on each plant and it becomes increasingly difficult to sample populations from each root system. Attributing a value to nodule occupancy by any strain requires careful selection of representative sample of nodules from plant roots. One issue with nodulation is that not all nodules contain live rhizobia, and this may not be known until after DNA analysis or attempts at plate culturing because the nodule may be destroyed otherwise. Both methods are time consuming, and depending on source materials, both can be expensive. It must be considered whether nodules are actually unoccupied or if experimental error contributed to improper data collection. Keeping unoccupied nodule counts in the analysis provided information about the rate of failed nodulation by the inoculant strains and how receptive vetch was to nodulation in general. As the number of
analyzed nodules increases, the number of failed nodulation events or unsuccessful plate cultures is also likely to increase in results.

It was interesting that unknown rhizobia DNA was found mostly in treatment combinations of three and four rhizobia strains because rhizobia mixing in indeterminate nodules has been previously reported (Bogino et al., 2011). If rhizobia strains mix in nodule tissue as suggested, evaluating inoculants on legumes with indeterminate nodules would be even more difficult and analyses of DNA fingerprints may need to account for similarities to multiple inoculant strains rather than try to specifically match one strain. Strain mixing in nodules may explain the low recovery of inoculant strains because rhizobia DNA mixing decreases detectability of individual strains. It may also be necessary to take extra steps to purify DNA in order to improve DNA fingerprint resolution from nodule extracts (Santasup, 2000). Inaccurate determination of nodule occupancy based on DNA identification will likely decrease as the technology and techniques used to identify rhizobia improve (Thies et al., 2001; Menna et al., 2009). Information collected in the experiment proved sufficient for an effective analysis of the four strains, but if more strains are used more resolution would be needed. It is recommended that assessment of strains for nodule occupancy occur in laboratory conditions where rhizobia can be more closely controlled and tracked after inoculation.

References cited


## Appendix A

Table A.1 Data collected for all vetch plants relating to N content and nodulation. Unit names: N = negative-N, P = positive-N, A = C10, B = NCSU332, C = NCSU435, D = NCSU478, and numbers represent reps.

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<th>Total N</th>
<th>BNF</th>
<th>Nodules counted</th>
<th>Nodule dry mass</th>
<th>C10 occ.</th>
<th>NCSU 332 occ.</th>
<th>NCSU 435 occ.</th>
<th>NCSU 478 occ.</th>
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<th>Not amplified</th>
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